THE INFLUENCE OF PLANT ROOTS ON AUTOTROPHIC NITRIFYING BACTERIA[1]

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Abstract

An investigation of the effects of corn and alfalfa roots on *Nitrosomonas* and *Nitrobacter* showed a stimulation of these organisms in the rhizosphere at 15 days but not at 46 days. A rapid method of estimating *Nitrosomonas* populations in soils was developed which depends on the color change in brom-thymol blue with the fall in pH associated with nitrite production.

In liquid media alfalfa root exudate inhibited *Nitrosomonas* at high concentrations but had no effect on the growth of *Nitrobacter*. Corn root exudate also inhibited *Nitrosomonas* but its effect on *Nitrobacter* was to reduce the duration of the lag phase without changing the generation time.

Introduction

Although considerable research has been conducted on the heterotrophic microflora associated with plant roots, little has been done on the influence of roots on the autotrophic nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*.

Goring and Clark (4) working with eight plant species and sampling at 5, 9, and 13 weeks reported no stimulation of *Nitrosomonas* or *Nitrobacter* around plant roots and suggested that the intensive development of heterotrophic microorganisms immobilized available nitrogen. Katznelson reported a slight stimulation of nitrifiers with mangels at the early stages (6) but with wheat there was no effect of roots upon nitrifying bacteria (7). Rivière (8) found an increase in *Nitrosomonas* and *Nitrobacter* in the "inner rhizosphere" of wheat at tillering and flowering. The findings of Theron (9) contradict those of Goring and Clark for he concluded that the reduction in nitrate in the rhizosphere was not due to immobilization but to inhibition of the nitrifying bacteria by roots.

In view of these conflicting reports it was decided to investigate the effects of roots on nitrifying bacteria in soil and in liquid media.

Methods

Plants and Soil

Two plant species were used: corn (*Zea mais*, Cornell hybrid M4) and alfalfa (*Medicago sativa* var. Dupuits).

The soil was a moderately well-drained gray-brown podzolic Lima silt loam with 6% organic matter, a base exchange capacity of 18 meq per 100 g and a pH ($CaCl_2$) of 7.0.

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Germinated alfalfa and corn seed was planted in separate pots containing 1700 g soil. The pots were immersed in a water bath which kept the soil temperature of 24° C ± 3° while the mean air temperatures of the greenhouse were 32° C and 17° C for the maximum and minimum respectively. The water content of the soil was adjusted each morning to 20% by weight on an oven-dry basis.

The rhizosphere samples, made up of the roots and firmly adhering soil, were introduced into 100 ml sterile de-ionized water and shaken for 15 minutes. The amount of soil in the rhizosphere samples was estimated by weighing the soil in a 2 ml aliquot of the suspension; the accuracy of this method was checked using known weights of soil in 100 ml water. The dry weight of roots in each rhizosphere sample was recorded. Non-rhizosphere or "control" soil samples were obtained from unsown pots kept under conditions similar to those for the sown pots. Between five and nine replicate soil or root samples were used for each count.

The presence of *Nitrosomonas* was detected by two methods: (*a*) The appearance of nitrite as shown with Tromsdorff's reagent in Winogradsky's medium: KH_2PO_4, 5 g; $MgSO_4.7H_2O$, 2.5 g; NaCl, 2.5 g; $Fe_2(SO_4)_3$, 0.05 g; $MnSO_4$, 0.05 g; $(NH_4)_2SO_4$, 0.5 g; $CaCO_3$, 7.5 g; de-ionized water 1 liter and partially sterilized at 80° C for 10 minutes. (*b*) The change in indicator color as the pH fell with nitrite production; this medium was identical with that of Engel and Alexander (3), modified by the addition of brom-thymol blue. Brom-thymol blue at a concentration of 0.002% was selected after testing several indicators at different concentrations.

The presence of *Nitrobacter* was indicated, again using Tromsdorff's reagent, by the disappearance of nitrite from two media: (*a*) Winogradsky's medium (K_2HPO_4, 5 g; $MgSO_4.7H_2O$, 2.5 g; NaCl, 2.5 g; $Fe_2(SO_4)_3$, 0.05 g; $MnSO_4$, 0.05 g; KNO_2, 6 mg; $CaCO_3$ 1 g; and water 1 liter); (*b*) Winogradsky's medium modified by replacing the $CaCO_3$ with K_2CO_3 to give a final pH of 8.6 after sterilization to give a particle-free medium.

Corn root exudate and alfalfa root exudate were collected from 1500 and 1700 plants respectively. The corn seeds were planted in batches of 500 on a 31×41 cm sheet of stainless steel mesh (5 mm holes) fitted in a plastic tray containing 5 liters of nutrient solution. With this arrangement the corn could be grown for 5 to 6 weeks and considerable root exudate collected although asepsis was not possible. Vigorous aeration of the root systems was necessary

for healthy growth. One hundred alfalfa seeds were grown on stainless steel mesh (1.5 mm holes) in 15 cm diameter glazed pots containing 600 ml solution, 17 such pots being set up.

The root exudate of corn and alfalfa was obtained by growing the plants in full strength plant nutrient solution of Hoagland and Arnon (5) and on alternate days transferring them to de-ionized water following an intermediate rinse in de-ionized water to free the roots of salts. Only the exudate which accumulated in the de-ionized water was used, as this could be concentrated without plant nutrient salts being present.

The root exudate solution was concentrated at 42° C under vacuum in a rotary film (flash) evaporator and sterilized by filtration through a sintered glass filter. This solution was added to sterile *Nitrosomonas* and *Nitrobacter* media to give two concentrations of exudate: (*a*) "high"—this contained the exudate from one plant in each 2 ml of medium; (*b*) "low"—this contained the exudate from one plant in each 10 ml of medium. *Nitrosomonas* was grown in the medium of Engel and Alexander (3) containing brom-thymol blue and *Nitrobacter* in the same medium except that 0.2% KNO_2 replaced the $(NH_4)_2SO_4$ and the pH was adjusted to 8.0. The media were dispensed with 15 ml per 15×150 mm tube and, after inoculation, were incubated at 28° C with rotary shaking. Aliquots of 0.1 ml were withdrawn at intervals and analyzed for nitrite by the α-naphthylamine method of Csaky (2). The effect of root exudate on the organisms was measured by the rate of nitrite appearance for *Nitrosomonas* and nitrite disappearance for *Nitrobacter*.

Results

Comparison of Media for Nitrosomonas and Nitrobacter

In a comparison of the numbers of *Nitrosomonas* in triplicate soil samples as estimated with Winogradsky's medium and brom-thymol blue medium it was found that the latter gave higher counts for each sample when incubated for 3 weeks or longer (Table I).

As it was possible that the acid production responsible for the color change in the brom-thymol blue may have been due to factors other than nitrite production, all the brom-thymol blue tubes were tested for nitrite at 3 weeks. From Table II it can be seen that all of the 463 tubes in which acid was produced contained nitrite. However, there were 25 tubes in which nitrite could be detected but no color change had occurred; this generally occurred at the

TABLE I

Number of *Nitrosomonas* per gram of soil as estimated with Winogradsky's and brom-thymol blue media

Weeks of incubation	Winogradsky's medium			Brom-thymol blue medium		
	1	2	3*	1	2	3*
2	95	29	59	59	29	59
3	590	550	590	2000	4000	1700
5	2900	2000	2900	4000	4000	4000

*Triplicate soil samples.

higher dilutions where the inoculum received by each tube was low and the development of acidity would be slow. In another series of experiments with pure cultures of *Nitrosomonas* and *Nitrobacter* it was found that neither the length of the lag phase nor the generation time was altered when bromthymol blue was incorporated into the medium.

Table II shows that the particle-free medium developed for estimating the numbers of *Nitrobacter* was unsatisfactory when compared with Winogradsky's medium, probably because of inadequate calcium and unsuitable pH.

Numbers of Bacteria in the Rhizosphere

Although estimates of the numbers of *Nitrosomonas* and *Nitrobacter* in the soil and the rhizosphere were based on the most probable number method with five replicate tubes from each dilution, large variations were found between

TABLE II

Number of positive tubes according to color (pH) change and nitrite formation (3 weeks incubation)

Soil	Dilution	No. tubes inoculated	Number of positive tubes			
			15*		46*	
			pH	NO_2^-	pH	NO_2^-
Control						
	10^{-2}	30	30	30	30	30
	10^{-3}	30	26	29	22	27
	10^{-4}	30	7	7	5	8
	10^{-5}	30	1	1	3	3
	10^{-6}	30	0	0	0	0
Rhizosphere						
Alfalfa	10^{-2}	40	40	40	40	40
	10^{-3}	40	28	28	16	19
	10^{-4}	40	14	15	3	3
	10^{-5}	40	2	2	0	0
	10^{-6}	40	1	1	0	0
Corn	10^{-2}	45	45	45	45	45
	10^{-3}	45	45	45	20	23
	10^{-4}	45	31	34	5	5
	10^{-5}	45	4	8	0	0
	10^{-6}	45	0	0	0	0

*Age of plants (days).

TABLE III

Comparative counts of *Nitrobacter* obtained on Winogradsky's medium and a particle-free medium (MPN/g soil)

Weeks of incubation	Winogradsky's medium			Particle-free medium†		
	1	2	3*	1	2	3*
1	290	290	290	28	28	28
2	5,900	15,700	15,700	28	28	28
3	95,000	40,000	95,000	290	280	280

*Triplicate samples of a soil.
†Made by substituting K_2CO_3 for $CaCO_3$ in Winogradsky's medium.

replicate soil samples. This large variability is shown in the standard deviations and fiducial limits presented in Table IV. In several instances the standard deviations for *Nitrosomonas* and *Nitrobacter* were over 50% of the mean.

TABLE IV

Numbers of heterotrophs, *Nitrosomonas*, and *Nitrobacter* per gram of control and rhizosphere soil

	Heterotrophs ($\times 10^6$)		*Nitrosomonas*		*Nitrobacter*	
Soil	15*	46*	15	46	15	46
Control						
N†	5	6	6	6	6	6
$\bar{x}$	82	71	4,017	4,138	403	240
$S\bar{x}$	7	4	1,344	2,021	62	80
F.L.	63	61	1,287	68	244	34
	101	81	6,747	8,208	562	446
Alfalfa rhizosphere						
N†	7	8	7	8	7	8
$\bar{x}$	840	370	17,743	7,800	4,414	273
$S\bar{x}$	230	70	5,514	4,660	1,165	127
F.L.	280	200	7,033	−1,040	1,563	−27
	1,400	540	28,453	16,640	7,265	573
Corn rhizosphere						
N†	9	9	9	9	9	9
$\bar{x}$	590	330	11,389	4,211	2,166	119
$S\bar{x}$	470	30	1,338	690	687	37
F.L.	−490	260	8,899	2,905	582	34
	1,670	400	13,879	5,517	3,760	204

*Age of plants (days).
†N = Number of replicate soil or rhizosphere samples.
$\bar{x}$ = Mean of N results.
$S\bar{x}$ = Standard deviation.
F.L. = Fiducial limits, based on $P = 0.05$ for heterotrophs and *Nitrobacter* and $P = 0.10$ for *Nitrosomonas*.

TABLE V

Numbers of heterotrophs, *Nitrosomonas*, and *Nitrobacter* per gram of roots

	Heterotrophs ($\times 10^7$)		*Nitrosomonas* ($\times 10^3$)		*Nitrobacter* ($\times 10^3$)	
Plant	15*	46*	15	46	15	46
Alfalfa						
N†	7	8	7	8	7	8
$\bar{x}$	46,143	469	1,109	114	314	4.12
$S\bar{x}$	11,327	61	281	70	123	2.28
F.L.	18,426	325	421	−52	13	−127
	73,860	613	1,797	280	615	9.51
Corn						
N†	9	9	9	9	9	9
$\bar{x}$	2,238	382	400	54	67.9	1.34
$S\bar{x}$	644	31	37	13	16	0.39
F.L.	753	311	315	24	31.2	0.44
	3,723	453	485	84	104	2.24

*Age of plants (days).
†N = Number of replicate rhizosphere samples.
$\bar{x}$ = Mean of N results.
$S\bar{x}$ = Standard deviation.
F.L. = Fiducial limits at $P = 0.05$.

The results indicate that a stimulation of *Nitrosomonas* and *Nitrobacter* occurred in the rhizospheres of both corn and alfalfa at 15 days but not at 46 days. The differences between the rhizospheres of corn and alfalfa were not significant at $P=0.05$ when the results were expressed on a soil weight basis (Table IV), but on a root weight basis at 15 days alfalfa supported more *Nitrosomonas* than did corn (Table V).

With the heterotrophs, numbers were higher in the rhizosphere samples and, as with the autotrophs, the 15-day-old roots supported higher populations than did 46-day-old plants when results were expressed on a root weight basis. On a soil weight basis, numbers did not show any significant difference between the 15- and 46-day-old samples.

Effects of Root Exudate

Root exudates collected from corn on 4 of its first 10 days of growth and from alfalfa on 12 of its first 21 days exerted quite different effects, depending on the plant and concentration of exudate.

Corn root exudate was toxic to *Nitrosomonas* (Fig. 1) causing a complete inhibition at the "high" concentration and strong inhibition at the "low" concentration. By contrast, *Nitrobacter* was stimulated by corn exudate which reduced the lag phase by 30 hours compared with the medium without root exudate although the generation time of 9 hours was not affected (Fig. 2).

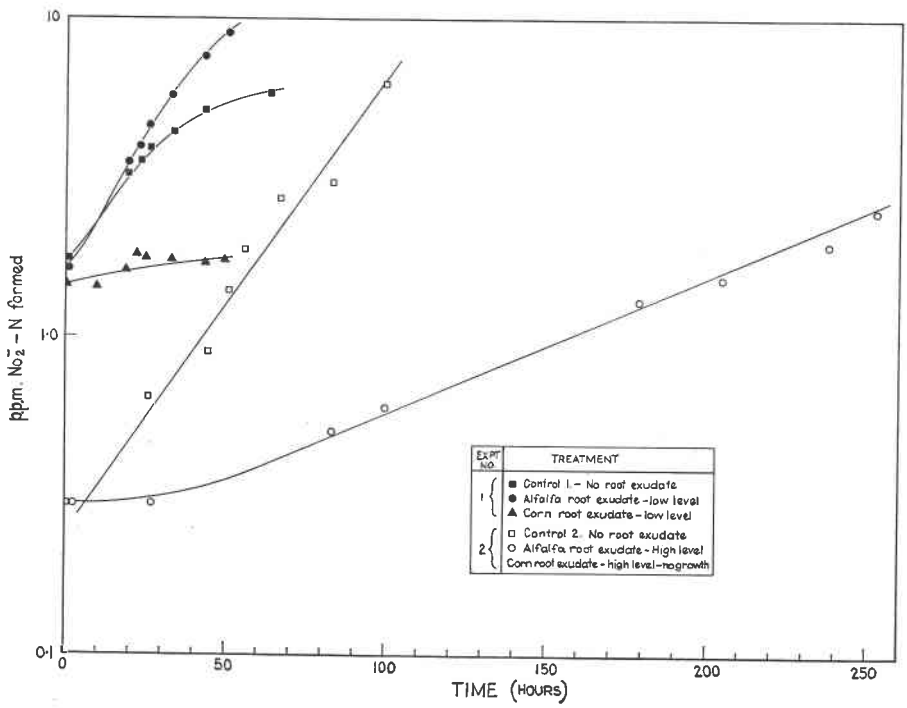

FIG. 1. Effect of corn and alfalfa root exudate on *Nitrosomonas*.

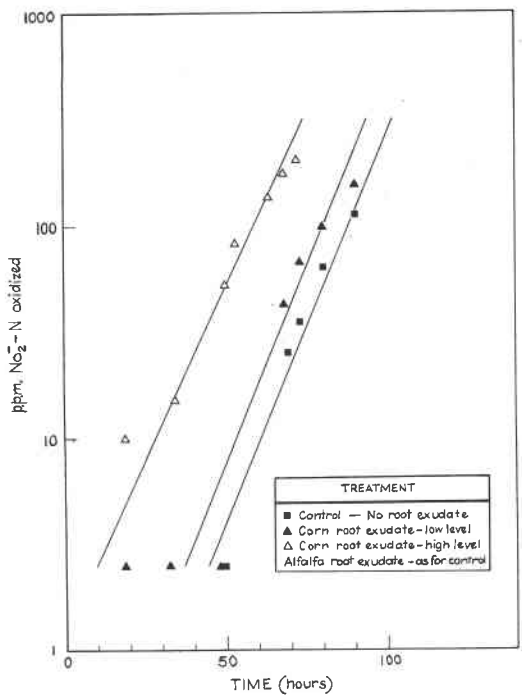

FIG. 2. Effect of corn and alfalfa root exudate on *Nitrobacter*.

Alfalfa root exudate appeared to have different effects on *Nitrosomonas* at the two concentrations (Fig. 1). At the "high" concentration it increased the lag phase by 100 hours and the generation time from 21 to 70 hours whereas at the "low" concentration it was slightly stimulatory. *Nitrobacter* was not affected by alfalfa exudate at either concentration.

Discussion

The close correlation between the formation of nitrite and pH change as revealed by brom-thymol blue and the similarity of the growth curves of *Nitrosomonas* with and without the indicator in the medium shows the value of the new medium for estimating the numbers of *Nitrosomonas* in soil. The indicator medium saves a considerable amount of time because a direct reading of the MPN tubes can be made instead of sampling at intervals and testing for nitrite as must be done with Winogradsky's medium. In the previous methods where the appearance of nitrite was used to indicate the presence of *Nitrosomonas* there was always the possibility that the nitrite may be oxidized to nitrate if *Nitrobacter* were present which would result in negative readings. This source of error does not arise when the production of acid is used as the indicator for the activity of *Nitrosomonas*.

Although the brom-thymol blue medium gave higher counts than did Winogradsky's medium, especially with 3 and 5 week incubation, there is still

no certainty that it is a true count of the numbers of *Nitrosomonas* in soil. In any comparison of numbers in different soil samples it should be remembered that the numbers obtained are relative rather than absolute.

Brom-thymol blue also proved of value as an indicator in the stock culture medium because it indicated when the cultures should be transferred to fresh media and reduced the risk of the cultures being killed by excess acidity.

The results indicate a discrepancy between soil studies and root exudate studies, for although 15-day plants in soil stimulated both *Nitrosomonas* and *Nitrobacter*, exudates collected over approximately the same period inhibited *Nitrosomonas* in pure culture. Probably these differences are due to the completely different environments. Even assuming that root exudate in soil is similar to exudate in liquid culture, the actual materials supplied to the autotroph will be different. In soil certain constituents of the exudate would be adsorbed by the clays while other materials would be altered by the active heterotrophic rhizosphere population, whereas in the exudate-collecting systems the materials released from the roots would be altered only slightly before being concentrated and sterilized. It is now well established that young plants exude considerable quantities of amino acids from their roots and the release of ammonia from these compounds by the heterotrophs would increase nutrient supply to *Nitrosomonas* and thence to *Nitrobacter* in the rhizosphere compared with soil distant from the root. In the experiments involving root exudates and pure cultures of the autotrophs the organisms would have been supplied with amino acids rather than ammonium.

The significance of root exudates in the rhizosphere compared with root cell debris declines as the plants develop, and this decline probably accounts for the observation that *Nitrosomonas* and *Nitrobacter* were stimulated by corn and alfalfa roots only at the first sampling.

A further difficulty of carrying out in vitro studies of the effects of root exudates upon microorganisms is that of using realistic concentrations. Extrapolation from in vitro results to in vivo conditions could be misleading unless a wide range of exudate concentrations is used. Nevertheless, pure-culture studies with root exudate supplements can indicate the potential of the exudate in influencing specific groups of organisms and are a necessary part of rhizosphere studies.

This investigation has shown that in the particular soil used for these experiments there was no evidence of inhibition of the autotrophic nitrifying organisms by the roots of corn or alfalfa; thus it supports the finding of Goring and Clark (4) that reduced nitrate levels in the rhizosphere are due to immobilization by the increased population of heterotrophs rather than an inhibition of *Nitrosomonas* or *Nitrobacter*.

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